Insulin Signaling After Exercise in Insulin Receptor Substrate-2–Deficient Mice

Kirsten F. Howlett, Kei Sakamoto, Michael F. Hirshman, William G. Aschenbach, Matthew Dow, Morris F. White, and Laurie J. Goodyear

The period immediately after exercise is characterized by enhanced insulin action in skeletal muscle, and on the molecular level, by a marked increase in insulin-stimulated, phosphotyrosine-associated phosphatidylinositol (PI) 3-kinase activity. Because the increase in PI 3-kinase activity cannot be explained by increased insulin receptor substrate (IRS)-1 signaling, the present study examined whether this effect is mediated by enhanced IRS-2 signaling. In wild-type (WT) mice, insulin increased IRS-2 tyrosine phosphorylation (~2.5-fold) and IRS-2–associated PI 3-kinase activity (~3-fold). Treadmill exercise, per se, had no effect on IRS-2 signaling, but in the period immediately after exercise, there was a further increase in insulin-stimulated IRS-2 tyrosine phosphorylation (~3.5-fold) and IRS-2–associated PI 3-kinase activity (~5-fold). In IRS-2−/− mice, the increase in insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity was attenuated as compared with WT mice. However, in IRS-2−/− mice, the insulin-stimulated, phosphotyrosine-associated PI 3-kinase response after exercise was slightly higher than the insulin-stimulated response alone. In conclusion, IRS-2 tyrosine phosphorylation and associated PI 3-kinase activity are markedly enhanced by insulin in the immediate period after exercise. IRS-2 signaling can partially account for the increase in insulin-stimulated phosphotyrosine-associated PI 3-kinase activity after exercise. Diabetes 51: 479–483, 2002

It is well established that skeletal muscle insulin sensitivity is enhanced after exercise (1–3) and subsequently affects a number of insulin-sensitive processes, such as glucose uptake (3–6), glycogen synthesis (4,7), and amino acid metabolism (8). The effects of exercise on insulin sensitivity and glucose homeostasis have important implications for the treatment and prevention of type 2 diabetes. However, the underlying mechanism(s) mediating the increase in muscle insulin sensitivity after exercise is equivocal (9). One hypothesis is that exercise or muscle contraction enhances insulin signaling downstream of the insulin receptor (10,11). The effects of insulin are mediated through the binding and activation of the insulin receptor, insulin receptor substrate (IRS)-1 and -2, and phosphatidylinositol (PI) 3-kinase (12). In contrast, an acute bout of exercise or muscle contractile activity, in situ or in vitro, does not increase tyrosine phosphorylation of the insulin receptor (6,10), IRS-1 (6,10,11), or IRS-2 (13). In addition, exercise does not affect IRS-1–associated or phosphotyrosine-associated PI 3-kinase activity (6,10,11). However, in the period immediately after exercise, insulin stimulation results in a marked increase in phosphotyrosine-associated PI 3-kinase activity as compared with the effect of insulin alone (6,11). This increase in PI 3-kinase activity cannot be explained by increased IRS-1–associated PI 3-kinase activity (6,11). Indeed, in the period after muscle contraction or exercise, insulin-stimulated, IRS-1–associated PI 3-kinase activity actually decreases (7,10) or remains unchanged (6,11). This suggests that there is another insulin-stimulated tyrosine phosphoprotein that binds and activates PI 3-kinase after exercise.

One possibility is that IRS-2 mediates the effect of prior exercise to increase insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity. In response to insulin, IRS-2 is rapidly phosphorylated in muscle, binds to the p85 subunit of PI 3-kinase, and activates the enzyme (14). Furthermore, studies in IRS-1−/− mice have provided evidence that IRS-2 may act as an alternative pathway of insulin action (14–16). In IRS-1−/− mice, insulin can increase glucose uptake, albeit to a lesser degree than in control animals (15), and results in a greater increase in IRS-2–associated PI 3-kinase activity compared with results in wild-type mice (16). Given the finding that IRS-2 can act as an alternative substrate for insulin signaling, we hypothesized that IRS-2 could be the tyrosine phosphoprotein that enhances insulin-stimulated PI 3-kinase activity after exercise.

In the present study, wild-type mice were examined to determine whether prior exercise results in a marked increase in insulin-stimulated IRS-2 tyrosine phosphorylation and IRS-2–associated PI 3-kinase activity. IRS-2−/− deficient mice were also examined to determine whether IRS-2 signaling mediates the effect of prior exercise to enhance insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity in skeletal muscle.

RESEARCH DESIGN AND METHODS

Animals. Male IRS-2−/− (IRS-2−/−) mice on a C57B16 × 129/Sv background and wild-type (WT) littermates were studied at approximately age 6 months. IRS-2−/− mice were produced by breeding homozygous IRS-2−/− male and female mice. Male IRS-2−/− mice (n=9) and WT mice (n=9) were fed a standard diet (Purina Rodent Chow) and were allowed to exercise on a treadmill as described below. Mice were killed by cervical dislocation, and gastrocnemius muscles were removed and immediately frozen in liquid nitrogen for assay of IRS-2 tyrosine phosphorylation.

Received for publication 20 June 2001 and accepted in revised form 1 November 2001.

2DG, 2-deoxy-glucose; IRS, insulin receptor substrate; MAP, mitogen-activated protein; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; WT, wild-type.
TABLE 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT (mmol/l)</th>
<th>IRS-2−/− (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>8.1 ± 0.4</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td>Exercise</td>
<td>6.4 ± 0.2*</td>
<td>7.5 ± 0.5†</td>
</tr>
<tr>
<td>Insulin</td>
<td>3.9 ± 0.3*</td>
<td>3.1 ± 0.3*</td>
</tr>
<tr>
<td>Exercise + Insulin</td>
<td>3.7 ± 0.3*</td>
<td>3.4 ± 0.5*</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 6–11 mice per group). *P < 0.05 vs. Rest; †P < 0.05 vs. WT.

5 weeks (IRS-2−/−, 49 ± 1 days; WT, 41 ± 1 days). The generation of these animals and many of the physiological characteristics of the mice have been described in detail previously (16,17). Only IRS-2−/− mice exhibiting fasting blood glucose levels similar to that of WT littermates were included in the study. Animals were housed under a 12-h light/dark cycle, having free access to standard laboratory diet and water. All protocols were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.

Experimental protocol. All mice were accustomed to the rodent treadmill (Quinton Instruments, Seattle, WA) by walking or running for 10 min/day for 2 consecutive days before the experiment. Mice were fasted for 12 h before the experiment. On the experimental day, mice were randomly divided into resting and exercise groups. Resting animals were injected intraperitoneally with insulin (0.15 units/g body wt) or an equal volume of saline. Immediately after exercise, the mice were injected intraperitoneally with insulin (0.15 units/g body wt) or an equal volume of saline. In the exercise group, the mice ran on the treadmill for 1 h at a speed of 22 m/min up a 10% incline. Immediately after exercise, the mice were injected intraperitoneally with either insulin or saline. In the control group, the mice ran on the treadmill for 1 h ta speed of 22 m/min up a 10% incline. Immediately after exercise, the mice were injected intraperitoneally with saline. The muscles were dissected after killing the mice, as described above. The muscles were glycogen analyzed and the remaining muscle was used for signaling measurements. The quadriceps and gastrocnemius muscle were quickly dissected and transferred to Krebs-Ringer bicarbonate buffer for 20 min, and then 2-deoxy-D-glucose (2DG) uptake was measured for 10 min at 30°C, as previously described (18). For insulin experiments, pork insulin (Lilly Research Laboratories, Indianapolis, IN) was used at a concentration of 50 μmol/l and was present throughout all incubations.

Glucose, insulin, and muscle glycogen. Blood glucose concentrations were measured using a glucose meter (One Touch II; LifeScan, Milpitas, CA). Serum insulin was assayed using a rat radioimmunoassay kit (Linco Research, St. Charles, MO). Muscle glycogen concentrations were measured as glycerol units after hydrolysis, as previously described (19).

Muscle preparation and signaling assays. Pulverized muscle was homogenized in Krebs-Ringer bicarbonate buffer for 20 min, and then 2-deoxy-D-glucose (2DG) uptake was measured for 10 min at 30°C, as previously described (18). For insulin experiments, pork insulin (Lilly Research Laboratories) was added to the muscle homogenate at a concentration of 50 μmol/l and was present throughout all incubations.

Muscle preparation and signaling assays. Pulverized muscle was homogenized in Krebs-Ringer bicarbonate buffer for 20 min, and then 2-deoxy-D-glucose (2DG) uptake was measured for 10 min at 30°C, as previously described (18). For insulin experiments, pork insulin (Lilly Research Laboratories) was added to the muscle homogenate at a concentration of 50 μmol/l and was present throughout all incubations.

Muscle preparation and signaling assays. Pulverized muscle was homogenized in Krebs-Ringer bicarbonate buffer for 20 min, and then 2-deoxy-D-glucose (2DG) uptake was measured for 10 min at 30°C, as previously described (18). For insulin experiments, pork insulin (Lilly Research Laboratories) was added to the muscle homogenate at a concentration of 50 μmol/l and was present throughout all incubations.

Muscle preparation and signaling assays. Pulverized muscle was homogenized in Krebs-Ringer bicarbonate buffer for 20 min, and then 2-deoxy-D-glucose (2DG) uptake was measured for 10 min at 30°C, as previously described (18). For insulin experiments, pork insulin (Lilly Research Laboratories) was added to the muscle homogenate at a concentration of 50 μmol/l and was present throughout all incubations.

Muscle preparation and signaling assays. Pulverized muscle was homogenized in Krebs-Ringer bicarbonate buffer for 20 min, and then 2-deoxy-D-glucose (2DG) uptake was measured for 10 min at 30°C, as previously described (18). For insulin experiments, pork insulin (Lilly Research Laboratories) was added to the muscle homogenate at a concentration of 50 μmol/l and was present throughout all incubations.
IRS-1 tyrosine phosphorylation was similar at rest between WT and IRS-2\(^{-/-}\) mice (Table 2). Treadmill exercise had no effect on IRS-1 tyrosine phosphorylation (Table 2). Insulin increased IRS-1 tyrosine phosphorylation by approximately twofold in both WT and IRS-2\(^{-/-}\) mice. The increase in insulin-stimulated IRS-1 tyrosine phosphorylation was not affected by prior exercise. IRS-1 protein levels were similar between groups (data not shown).

In WT mice, exercise had no effect on IRS-2 tyrosine phosphorylation, as levels were similar to basal (Fig. 1). In response to insulin, IRS-2 tyrosine phosphorylation increased \(\sim 2.5\)-fold. Prior exercise further increased insulin-stimulated IRS-2 tyrosine phosphorylation, with levels augmented to \(~3.5\)-fold above resting levels. IRS-2–associated PI 3-kinase activity in WT mice followed a trend similar to the IRS-2 tyrosine phosphorylation data (Fig. 2). Exercise had no significant effect on IRS-2–associated PI 3-kinase activity, whereas insulin-stimulated, IRS-2–associated PI 3-kinase activity increased approximately threefold above resting levels. The effect of prior exercise on insulin-stimulated, IRS-2–associated PI 3-kinase activity resulted in a further increase (fivefold above resting levels) when compared with the effect on insulin-stimulated activity alone. These results demonstrate that prior exercise results in a marked increase in insulin-stimulated IRS-2 signaling.

IRS-2–deficient mice were examined to determine whether IRS-2 is necessary for prior exercise to increase insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity. Phosphotyrosine-associated PI 3-kinase activity was similar between WT and IRS-2\(^{-/-}\) mice at rest and was unchanged by treadmill exercise (Fig. 3). In WT mice, phosphotyrosine-associated PI 3-kinase activity was increased in response to insulin by 6- to 7-fold, and prior exercise further increased insulin-stimulated phosphotyrosine-associated PI 3-kinase activity to 10-fold above basal levels. In IRS-2\(^{-/-}\) mice, insulin increased phosphotyrosine-associated PI 3-kinase activity; however, this response was attenuated in IRS-2\(^{-/-}\) mice as compared with WT mice. In IRS-2\(^{-/-}\) mice, prior exercise resulted in a small, but nonsignificant, increase \((\sim 30\%)\) in insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity compared with insulin-stimulated activity alone. These findings suggest that IRS-2 signaling can only partially account for the marked increase in insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity observed after exercise in WT mice.

**2DG uptake in skeletal muscle.** At rest, glucose uptake was similar between WT and IRS-2\(^{-/-}\) mice \((1.53 \pm 0.22\) vs. \(1.73 \pm 0.33\) \(\mu\)mol \(\cdot\) g\(^{-1}\) \(\cdot\) h\(^{-1}\), respectively). In response to either exercise or insulin, glucose uptake increased above resting levels. The combination of exercise plus insulin further augmented the increase in glucose uptake in WT \((P < 0.05\) compared to insulin) and IRS-2\(^{-/-}\) mice \((P = 0.053\) compared with insulin) (Fig. 4). The measured increase in insulin-stimulated glucose uptake after exercise was greater than would be predicted from the additive effects of the individual stimuli.
DISCUSSION

It has been suggested that the mechanism mediating the effect of prior exercise to increase insulin sensitivity is a result of enhanced insulin signaling downstream of the insulin receptor. In the present study, prior exercise had no effect on insulin-stimulated insulin receptor tyrosine phosphorylation compared with insulin stimulation alone. However, in agreement with previous studies (6,11), there was a marked increase in phosphotyrosine-associated PI 3-kinase activity with insulin after exercise. Insulin-stimulated, IRS-1–associated PI 3-kinase activity is not increased after exercise (6,7,10,11) and subsequently cannot account for the increase in phosphotyrosine-associated PI 3-kinase activity after exercise.

The results from the present study in WT mice demonstrate that exercise does not affect IRS-2 signaling. However, prior exercise did result in an augmented response in insulin-stimulated IRS-2 tyrosine phosphorylation and IRS-2–associated PI 3-kinase activity compared with insulin stimulation alone. The underlying mechanism mediating this increase in insulin-stimulated IRS-2 signaling in the period immediately after exercise is unknown, although it appears that there is specificity to IRS-2, as insulin-stimulated IRS-1 tyrosine phosphorylation after exercise was unchanged compared with insulin stimulation alone. It is also apparent that the enhanced insulin-stimulated IRS-2 signaling response is specific to in vivo exercise (Figs. 1 and 2), as insulin-stimulated IRS-2– and phosphotyrosine-associated PI 3-kinase activity were not increased with ex vivo contractions in rat epitrochlearis muscle (21). A recent study has also demonstrated that insulin-stimulated IRS-2–associated PI 3-kinase activity increased 16 h after prolonged swimming exercise in rats (22). However, it should be noted that in that study (22), IRS-2 protein expression was also increased, which may have accounted for the enhanced PI 3-kinase activity. It has been suggested that a serum factor, probably a protein, is required for enhanced insulin sensitivity after muscle contraction (23). Indeed, in one study, when intact muscle was isolated, washed, and contracted in vitro, there was no increase in insulin sensitivity (24). In light of these findings, it is possible that a serum factor could be responsible for the increase in insulin-stimulated IRS-2 signaling after exercise.

Although prior exercise results in a marked increase in insulin-stimulated IRS-2 signaling in WT mice, it appears that IRS-2 may only partially account for the marked increase in insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity after exercise. In IRS-2–deficient mice, insulin-stimulated phosphotyrosine-associated PI 3-kinase activity was attenuated compared with WT mice. However, in IRS-2–deficient mice, there was a small increase in phosphotyrosine-associated PI 3-kinase activity with the combination of prior exercise and insulin, as compared with after insulin alone. This raises the possibility that there is another tyrosine phosphoprotein in addition to IRS-2 that binds and activates PI 3-kinase in response to insulin after exercise.

In isolated cardiomyocytes, immunoprecipitation of p85α revealed a markedly enhanced tyrosine phosphorylation of an unknown co-precipitated 200-kDa protein in response to both insulin and contraction (25). However, after analysis of tyrosine-phosphorylated proteins in p85 immunoprecipitates, we could not identify this 200-kDa protein in exercise- and/or insulin-stimulated skeletal muscle, nor could we identify any other tyrosine-phosphorylated protein apart from IRS-2 that was upregulated with the combination of prior exercise and insulin (data not shown). Although the detection of additional phosphoproteins in muscle has been unsuccessful thus far, it is likely that there are limitations in sensitivity at the protein level.

As previously demonstrated (17), euglycemic IRS-2–deficient mice have a normal glucose transport response at rest and after insulin stimulation and/or exercise. However, an interesting observation in the present study was that glucose transport was normal despite a significant reduction in insulin-stimulated, phosphotyrosine-associated PI 3-kinase activity in IRS-2–deficient mice. This finding suggests that there is redundancy with regard to the insulin-signaling pathway mediating glucose transport. Furthermore, there appears to be no significant compensatory effect on IRS-1 tyrosine phosphorylation in IRS-2–deficient mice. Because other IRS proteins, such as IRS-3 (26) and IRS-4 (27), are not expressed in adult skeletal muscle, signaling through IRS-1 is likely to be sufficient in mediating the effect of insulin on muscle glucose transport. It appears that in vivo, IRS-1 has a more important role than IRS-2 in the regulation of insulin-stimulated muscle glucose transport (17,28). The results from this study also demonstrated that with insulin stimulation after exercise, there was synergistic activation of glucose transport in muscle from WT and IRS-2–deficient mice. The synergistic effect on glucose transport has been shown previously (6,17); furthermore, based on results in muscle-specific insulin receptor knockout mice, it appears that this effect is independent of the insulin receptor (6). It remains to be determined whether insulin can bind to receptors other than the insulin receptor and/or activate

FIG. 4. 2DG uptake in soleus muscle in vitro. Glucose uptake is presented as increase above rest (1.53 ± 0.22 [WT] vs. 1.72 ± 0.33 [IRS-2−/−] μmol · g−1 · h−1). Data are means ± SE (n = 6–11 mice per group). Ex+Ins, combination of exercise and insulin; Ex+Ins calculated, sum of Exercise and Insulin. *P < 0.05 compared with Rest; †P < 0.05 compared with Insulin.
other signaling pathways. Alternatively, exercise could induce relocalization of insulin-signaling molecules so that more substrates are available for activation by insulin (6). Exercise may also increase the susceptibility of muscle glucose transport to activation by stimuli other than insulin (24). It is important to note that in this study, insulin signaling and glucose uptake were studied in response to supraphysiological concentrations of insulin. Therefore, the combined effects of exercise and insulin under true physiological insulin concentrations are not known.

There may be additional physiological responses, other than glucose transport, that could be mediated by the increase in insulin-stimulated, IRS-2– or phosphotyrosine-associated PI 3-kinase activity in skeletal muscle after exercise. Muscle glycogen synthesis is likely to be regulated by PI 3-kinase activity, as PI 3-kinase activity has been shown to correlate with enhanced GSK-3α Ser21 phosphorylation after insulin stimulation in exercised muscle (6). Little is known about the signaling mechanisms mediating amino acid transport, but prior exercise does result in an increase in insulin-stimulated amino acid transport in muscle (8). It is also possible that enhanced IRS-2 signaling regulates gene transcription and protein synthesis via the mitogen-activated protein (MAP) kinase signaling pathways. However, in contrast to the effects on PI 3-kinase activity, preliminary results from our laboratory suggest that MAP kinase signaling does not exhibit additive or synergistic effects with the combination of insulin and exercise (K.F.H., L.J.G., unpublished observations).

In conclusion, prior exercise results in a marked increase in insulin-stimulated IRS-2 tyrosine phosphorylation and IRS-2–associated PI 3-kinase activity in mouse skeletal muscle. The results from IRS-2–deficient mice demonstrate that IRS-2 is one of the tyrosine phosphoproteins that mediates the insulin-stimulated increase in PI 3-kinase activity after exercise. The results also suggest that in addition to IRS-2, there is another, unknown, tyrosine-phosphorylated protein that mediates the effect of prior exercise to enhance insulin signaling. Elucidating the underlying mechanism(s) that mediates IRS-2 signaling will be important in further understanding the molecular basis for the beneficial effects of exercise on increasing insulin sensitivity in skeletal muscle.

ACKNOWLEDGMENTS

This study was funded by National Institutes of Arthritis and Musculoskeletal and Skin Diseases Grants AR-45670 and AR-42238 (L.J.G.) and by the National Institutes of Health Grant DK-43808 (M.F.W.). K.F.H. was supported by a Fulbright postdoctoral award. W.G.A. was supported by a NSRA grant (DK59769).

REFERENCES